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Insect Biochemistry and Molecular Biology 37 (2007) 97–106
Insect Biochemistry and Molecular Biology
www.elsevier.com/locate/ibmb

Binding of *Bacillus thuringiensis* toxin Cry1Ac to multiple sites of cadherin in pink bollworm

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Received 14 July 2006; received in revised form 15 September 2006; accepted 20 October 2006

Abstract

Toxins from *Bacillus thuringiensis* (Bt) are widely used for pest control. In particular, Bt toxin Cry1Ac produced by transgenic cotton kills some key lepidopteran pests. We found that Cry1Ac binds to recombinant peptides corresponding to extracellular regions of a cadherin protein (BtR) in a major cotton pest, pink bollworm (*Pectinophora gossypiella*) (PBW). In conjunction with previous results showing that PBW resistance to Cry1Ac is linked with mutations in the *BtR* gene, the results reported here support the hypothesis that BtR is a receptor for Cry1Ac in PBW. Similar to other lepidopteran cadherins that bind Bt toxins, BtR has at least two Cry1Ac-binding domains in cadherin-repeat regions 10 and 11, which are immediately adjacent to the membrane proximal region. However, unlike cadherins from *Manduca sexta* and *Bombyx mori*, toxin binding was not seen in regions more distal from the membrane proximal region. We also found that both the protoxin and activated toxin forms of Cry1Ac bound to recombinant BtR fragments, suggesting that Cry1Ac activation may occur either before or after receptor binding.

Keywords: Cadherin; BtR; *Pectinophora gossypiella*; Pink bollworm; Cry1Ac receptor; *Bacillus thuringiensis* (Bt)

1. Introduction

The bacterium *Bacillus thuringiensis* (Bt) produces insecticidal crystal (Cry) proteins used in sprays and transgenic crops for pest control. A critical step in the mode of action of Cry proteins is binding of toxin to midgut membrane receptors (Schnepf et al., 1998; Whalon and Wingerd, 2003). In Lepidoptera, several proteins bind Cry1A toxins and may mediate toxicity, including aminopeptidases, alkaline phosphatases, and cadherins (Knight et al., 1994; Sangadala et al., 1994; Gill et al., 1995; Jurat-Fuentes and Adang, 2004; Jurat-Fuentes et al., 2004; Vadlamudi et al., 1993, 1995; Nagamatsu et al., 1998).

Two lines of evidence support the hypothesis that cadherins are receptors for Cry1A toxins in some Lepidoptera. First, Cry1A toxins bind to cadherins in at least five species (Vadlamudi et al., 1995; Nagamatsu et al., 1999; Dorsch et al., 2002; Hua et al., 2004; Jurat-Fuentes et al., 2004; Flannagan et al., 2005; Wang et al., 2005; Xie et al., 2005). Second, mutations in genes encoding cadherin proteins are tightly linked with resistance to Cry1Ac in at least three pests: tobacco budworm, *Heliothis virescens* (Gahan et al., 2001), cotton bollworm, *Helicoverpa armigera* (Xu et al., 2005), and pink bollworm (PBW), *Pectinophora gossypiella* (Morin et al., 2003).

This paper examines binding of Cry1Ac to cadherin in PBW, which is a major pest of cotton in the southwestern United States and elsewhere (Ingram, 1994; Henneberry and Naranjo, 1998). Transgenic cotton producing Cry1Ac has been useful for controlling PBW since 1996 (Carrière et al., 2003; Tabashnik et al., 2005b). In several laboratory-selected strains of PBW, resistance to Cry1Ac is tightly linked to three recessive alleles of a gene encoding a cadherin protein called BtR (Morin et al., 2003; Tabashnik et al., 2004; Tabashnik et al., 2005a). Similar to other lepidopteran cadherins (Nagamatsu et al., 1998; Dorsch et al., 2002; Wang et al., 2005; Xie et al., 2005), the proposed structure of BtR includes an amino-terminal signal sequence, 11 cadherin repeats (CRs), a

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doi:10.1016/j.ibmb.2006.10.010

membrane-proximal region (MPR), a transmembrane domain, and a cytoplasmic domain (Morin et al., 2003) (Fig. 1A).

Given that cadherins in other Lepidoptera bind Cry1A toxins and that mutations in BtR are linked with resistance to Cry1Ac in PBW, binding of Cry1Ac to BtR is likely. More specifically, we hypothesized that Cry1Ac binds to the extracellular portion of BtR (amino acid residues 1–1152) as in other Lepidoptera (Nagamatsu et al., 1999; Dorsch et al., 2002; Hua et al., 2004; Wang et al., 2005; Xie et al., 2005). To test this hypothesis, we evaluated specific regions of PBW BtR for binding of Cry1Ac.

We produced and assessed recombinant fragments of BtR (rBtRs) to determine which, if any, portions of the protein bind Cry1Ac. This enabled us to test the hypothesis that the mutations linked with resistance to Cry1Ac in PBW occur in the regions of BtR that bind Cry1Ac. We also compared the toxin binding regions of BtR with those reported for cadherins from other Lepidoptera. We found that, similar to other lepidopteran cadherins, BtR contains at least two Cry1Ac binding sites in CR regions 10 and 11 near the MPR. Both the protoxin and trypsin-activated forms of Cry1Ac bound to these regions. Only one of the three mutant alleles linked with resistance (*r*2) is predicted to affect the amino acid residues in the Cry1Ac-binding regions of BtR.

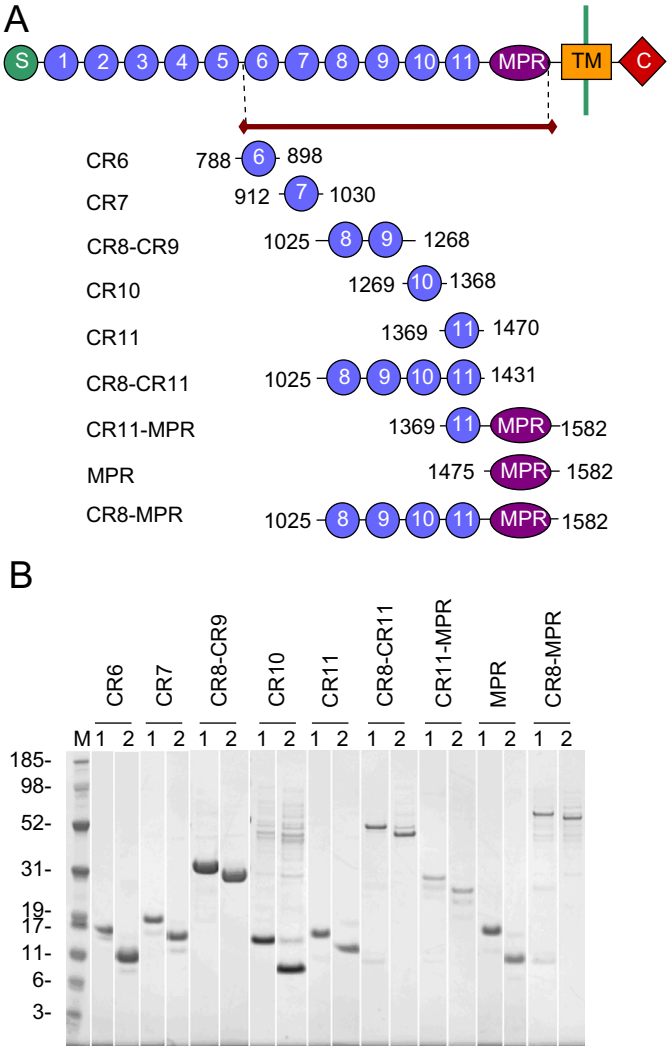

Fig. 1. Recombinant fragments of cadherin (rBtRs) from pink bollworm. (A) Schematic diagram of BtR with amino-terminal signal sequence (S), cadherin repeats (CRs) 1–11, membrane-proximal region (MPR), transmembrane region (TM), and cytoplasmic domain (CD). (B) rBtRs were expressed, purified, digested with rAcTEV protease, re-purified to remove six histidine tag and protease, and analyzed by SDS–PAGE. Undigested (1) and rAcTEV protease-digested rBtR fragments (2) were separated by SDS–PAGE and stained. Lane M shows protein molecular weight standards (kDa).

2. Materials and methods

2.1. Insects and cDNA synthesis

PBW larvae were reared in the laboratory on wheat germ diet (Bartlett and Wolf, 1985). We used the susceptible strain APHIS-S, which has been reared in the laboratory for >20 years without exposure to toxins (Tabashnik et al., 2000).

Total RNA was obtained from female third instar larvae ($n = 19$, 87.9 mg fresh weight) by homogenizing in TRIzol reagent (Invitrogen), extracting with chloroform, and subsequently precipitating with isopropanol and ethanol. The first strand cDNA synthesis was catalyzed from total RNA using either random hexamers or an oligo-dT primer and Superscript III M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Following cDNA synthesis, RNA was removed by treatment with RNase H.

2.2. Production of rBtRs

PCR and Platinum Pfx DNA polymerase (Invitrogen) was used to amplify products from cDNA corresponding to partial PBW BtRs.[1] PCR products were gel-purified and inserted into the *Escherichia coli* expression vector pET151-D-TOPO (Invitrogen). The following PCR primers were used to generate the BtR cDNA fragments: CR6, 17btr5 and 18btr3; CR7, 13btr5 and 14btr3; CR8–CR9, 1btr5 and 4btr3; CR10, 2btr5 and 5btr3; CR11, 3btr5 and 15btr3; CR11-MPR, 3btr5 and 6btr3; MPR, 16btr5 and 6btr3; CR8–CR11 and CR8-MPR, 1btr5 and 6btr3 (Table 1). The CR8–CR11 fragment was inadvertently obtained as a result of the spontaneous change of C to T at base 4293, resulting in the introduction of a premature stop codon at residue 1431. Plasmids containing BtR constructs were propagated in TOP10 OneShot *E. coli* (Invitrogen) and using Qiagen's Plasmid Midi Kit. Insertion of the correct sequence into each expression vector was confirmed by sequencing plasmids in both directions using T7 and T7 reverse vector primers.

[1]Mention of a commercial or proprietary product does not constitute a recommendation by the USDA. The complete amino acid sequence of PBW BtR can be accessed through NCBI Database under NCBI Accession # AY198374.

Table 1
PCR primers used to generate rBtR cDNA fragments

Primer	Primer DNA sequence
1btr5	5′-CACCGGTATAACACTCGATGGCGAA-3′
2btr5	5′-CACCAGGTTCTCGCTCCCACATGCA-3′
3btr5	5′-CACCACGGCTGGCATTTCCACTTC-3′
4btr3	5′-CTACTCAGTCAAGCCAACCTCTTTTTC-3′
5btr3	5′-CTAGTACAGACGCTGCTCGAACACAGG-3′
6btr3	5′-CTAGATGGTGATCTGCACGCC-3′
13btr5	5′-CACCTACTCCATCGACGAAGACAGA-3′
14btr3	5′-CTAGCCATCGAGTGTTATACCCTCTTGT-3′
15btr3	5′-CTAGAAGGACACCCTATTTTGGGATG-3′
16btr5	5′-CACCCTGAACGATGTGGAGACGGTTGA-3′
17btr5	5′-CACCCTCGCTACAGACATTGATGG-3′
18btr3	5′-CTAAATGTACACCGTTTCGTTGAATCGA-3′

For expression of recombinant proteins, 10 ng of plasmid DNA was used to transform BL21 Star (DE3) *E. coli*. Transformed cells were grown overnight at 37 °C in two 5-mL cultures of LB containing 0.05 mg/mL carbenicillin. Cells were pelleted by centrifugation, resuspended in 5 mL of fresh LB broth, and used to inoculate one liter of LB broth containing 0.05 mg/mL carbenicillin. The cultures were grown at 37 °C until an OD_{600} of 0.6 was reached. BtR protein expression was induced by the addition of IPTG at a final concentration of 0.001 M. The induced cultures were grown overnight at 25 °C with vigorous shaking. After cooling on ice, the cells were harvested by centrifugation. Cell pellets were washed with 0.02 M sodium phosphate, 0.5 M sodium chloride, pH 7.8 buffer, pelleted by centrifugation, and stored at −80 °C.

2.3. Affinity purification of recombinant BtRs

Because the pET151-D-TOPO expression vector produces recombinant protein containing an amino-terminal six-histidine tag, Ni^{2+}-affinity chromatography was used to purify rBtRs. Frozen cell pellets were thawed on ice and resuspended in guanidinium lysis buffer (6 M guanidine hydrochloride, 0.02 M sodium phosphate, 0.5 M sodium chloride, pH 7.8). Cells were lysed by slowly rocking for 10 min at 25 °C in lysis buffer, followed by three, 5 s pulses with a sonicator on ice. The clear-lysate supernatant was collected following centrifugation. Two mL of Ni-NTA agarose resin (Invitrogen) was equilibrated in buffer containing 8 M urea and chromatography was conducted by batch elution under hybrid conditions and following the purification protocol as outlined in Ni-NTA user manual (Invitrogen).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted to confirm presence of purified rBtRs. Elution fractions containing rBtRs were pooled and dialyzed against 0.01 M Tris–HCl, 0.01% Triton X-100, pH 8.0, with two separate buffer changes.

2.4. Protein concentration

Centricon-10 centrifugal filters (Millipore) were used to concentrate pooled elution samples containing rBtRs. The amount of rBtR recovered from affinity chromatography was determined with Coomassie plus protein assay reagent (Pierce) with bovine serum albumin as a standard.

2.5. Proteolytic removal of amino-terminal tag

Recombinant proteins expressed from the pET151-D-TOPO vector contain 33 additional amino acid residues at their amino-termini, including a six-histidine tag and a TEV cleavage site. The TEV recognition site allows for removal of the amino-terminal fusion tag from the recombinant BtR fusion protein. A total of 0.65 mg of each rBtR was digested with 65 units of rAcTEV protease (Invitrogen) for 4 h at 30 °C. Since the rAcTEV protease contains a six-histidine tag, both it and the six-histidine cleavage products were separated from the rBtRs by retention on Ni-NTA agarose resin. The purified rBtR proteins contain an additional six residues (GIDPFT) at their amino termini.

SDS–PAGE was conducted to confirm cleavage of rBtRs and removal of rAcTEV protease. Elution fractions containing rBtRs were pooled and protein concentration was determined as described above.

2.6. Proteolytic activation and purification of Cry1Ac

Soluble recombinant Cry1Ac protoxin was prepared from transformed *E. coli* cells and was provided by Luke Masson, Biotechnology Research Institute of the National Research Council, Montreal. Activated toxin was prepared by incubating with 10:1(w/w) of protoxin:TPCK-treated bovine trypsin (Sigma) for 2 h at 37 °C. The digested protein sample was centrifuged and the pH of the supernatant was adjusted to 9.5–10.0. Purified toxin was obtained from a HiTrap Q HP anion exchange column (Amersham) eluted with a linear sodium chloride gradient (0–0.6 M NaCl). Pooled fractions containing activated toxin (as determined by SDS–PAGE and immunoblot analysis) were concentrated and the buffer exchanged with 0.01 M Tris–HCl pH 8.0 by using a Centricon-10 filter.

2.7. Polyclonal antibodies against rCR8–CR9, rCR8–CR11, and rCR8-MPR

Sera from New Zealand white rabbits were prescreened by immunoblot analysis for low initial cross-reactivity against rBtRs. rTEV-Protease-treated rCR8–CR9, rCR8–CR11, and rCR8-MPR in 10 mM Tris–HCl, pH 8.0 were mixed with Freund's adjuvant and injected into individual animals by Cocalico Biologicals, Inc. according to their standard protocol.

2.8. Dot blot binding of rBtRs to Cry1Ac: toxin and rBtR overlay

To test regions of BtR for binding of Cry1Ac toxin, we used two sets of dot blot binding assays under nondenaturing conditions: toxin overlays and rBtR overlays. In toxin overlays, rBtRs were immobilized on membranes, Cry1Ac was added, and binding was detected with Cry1Ac antibody. In rBtR overlays, Cry1Ac was immobilized on membranes, rBtRs were added, and binding was detected with rBtR-specific antibodies. Rabbit antiserum made against Cry1Ac protoxin was provided by Anthony Pang from the Great Lakes Forestry Centre of the Canadian Forestry Service, Sault Ste. Marie, Ontario.

In both sets of dot blot assays, the immobilized material (0.01, 0.1, 0.5, or 1.0 μg of rBtR or Cry1Ac) was diluted in PBS (0.08 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.1 M NaCl, pH 7.4) and spotted onto Immobilon-P PVDF membrane (Millipore). After drying, membranes were blocked in 3% BSA in PBS for 1 h. Incubations with target ligands were done for 2 h in PBS, pH 7.4, 0.1% BSA, 0.3% Tween-20. Blots were washed between each step three times for 5 min in wash buffer (PBS buffer, pH 7.4, 0.2% BSA, 0.1% Tween-20). Both sets of assays used rabbit polyclonal antisera to detect binding. Primary antibody binding was detected with ECL-horseradish peroxidase-labeled anti-rabbit antibody (Amersham) diluted 1:5000 in wash buffer. ECL Western blotting detection reagent (Amersham) was used to visualize peroxidase activity on a FluorChem imager (Innotech). All steps were carried out at room temperature on an orbital shaker.

In the toxin overlays, membranes with immobilized rBtRs were incubated with 10 nM Cry1Ac protoxin or trypsin-activated toxin. After washes, bound Cry1Ac was detected with Cry1Ac antibody diluted 1:3000 in PBS wash buffer. In the rBtR overlays, membranes with immobilized Cry1Ac protoxin or trypsin-activated toxin were incubated with 10 nM rBtR (rCR8–CR9, rCR11, rCR8–CR11, rCR8-MPR, or rMPR). After washes, bound rBtRs were detected with anti-rCR8–CR9 for rCR8–CR9; anti-rCR8–CR11 for rCR11, rCR8-CR11; and anti-rCR8-MPR for rCR11-MPR, rCR8-MPR, and rMPR. We did not test rCR6, rCR7, and rCR10 by this method because our antisera did not cross-react with these peptides (Table 2).

2.9. SDS–PAGE and immunoblot analysis

For SDS–PAGE and immunoblots, proteins were denatured in 1x NuPAGE LDS sample buffer (Invitrogen) at 70 °C for 10 min. Proteins were either stained with SimplyBlue Coomassie SafeStain (Invitrogen) or elecroblotted onto nitrocellulose. For immunoblot analysis, the blots were blocked with 5% (w/v) nonfat dry milk in 1x TBS-T (0.02 M Tris–HCl, pH 7.6, 0.137 M NaCl, 0.1% Tween-20) and probed with rabbit antiserum against Cry1Ac, rCR8–CR9, rCR8–CR11, or rCR8-MPR (1:2000 dilution) in 1x TBS-T. Antibody binding was observed by a color reaction catalyzed by alkaline phosphatase conjugated to goat anti-rabbit IgG (BioRad). MultiMark protein molecular weight standards (Invitrogen) were run simultaneously.

Table 2
Recognition of rBtR fragments by rabbit polyclonal antisera[a]

BtR fragment	Antisera		
	α-rCR8-CR9	α-rCR8-CR11	α-rCR8-MPR
rCR6	−	−	−
rCR7	−	−	−
rCR8-CR9	+	+	+
rCR10	−	−	−
rCR11	−	±	−
rCR8-CR11	+	+	+
rCR11-MPR	−	−	+
rMPR	−	−	+
rCR8-MPR	+	+	+

[a]Degree of antibody recognition detected by immunoblot analysis and indicated as "−, +, or ±" for no cross-reactivity, strong cross-reactivity, and weak cross-reactivity, respectively.

2.10. Ligand blot binding assay of rBtRs to Cry1Ac

Toxin overlay assays were performed by using a protocol modified from Xie et al. (2005). One microgram of each rTEV protease-treated rBtR was separated by 10% SDS–PAGE and transferred to Immobilon-P PVDF membrane (Millipore). After blocking in 3% BSA in PBS, filters were incubated overnight with 10 nM Cry1Ac protoxin or 10 nM activated toxin in PBS, pH 7.4, 0.1% BSA, 0.3% Tween-20. Washes, incubations with antisera, and development of chemiluminescence were performed as previously described for dot blot binding assays and were conducted at room temperature with gentle shaking on an orbital shaker. Samples were not subjected to heat treatment before separation by SDS–PAGE. ECL Dual-Vue western blotting markers (Amersham) were run simultaneously and were detected by including S-protein-HRP conjugate with the secondary antibody incubation.

2.11. Binding of Cry1Ac to midgut brush border membrane vesicles

Brush border membrane vesicles (BBMV) were prepared by methods modified from Flannagan et al. (2005) and Wolfersberger et al. (1987). Midguts from fourth instar larvae (25♀ and 25♂) were dissected and homogenized in ice-cold MET buffer [0.3 M mannitol, 0.017 M Tris–HCl, pH 7.5, 0.005 M EGTA, Complete-mini EDTA-free protease inhibitor cocktail (Roche)]. An equal volume of 0.028 M $MgCl_2$ was added and homogenates were allowed to set for 15 min before centrifugation at 30,000 *g* for 30 min at 4 °C. The pellet was resuspended in MET buffer and centrifugation was repeated. The BBMV pellet was

Table 3
rAcTEV protease-treated rBtR fragments

rBtR	BtR residue position	Residue total	BtR sequence[b]	Predicted MW (kDa)[a]	
				Before	After
CR6	788–898	110	LATDIDG…RFNETVYIY	16.4	13.2
CR7	912–1030	118	YSIDEDRD…QEGITLDG	17.7	14.5
CR8–CR9	1025–1268	243	GITLDGE…EKEVGLTE	30.7	27.5
CR10	1269–1368	99	RFSLPHA…PVFEQRLY	14.9	11.8
CR11	1369–1470	101	TAGISTS…SQNRVSF	15.1	12.0
CR8–CR11	1025–1431	407	GITLDGE…TGVLILRI	48.5	45.4
CR11–MPR	1369–1582	213	TAGISTS…GVQITI	27.0	23.9
MPR	1475–1582	107	LNDVETVE…GVQITI	15.5	12.3
CR8–MPR	1025–1582	557	GITLDGE…GVQITI	65.0	61.9

[a]Molecular weights were calculated for each partial rBtR before and after treatment with rAcTEV protease by using Compute pI/MW from the ExPASy Molecular Biology Server.
[b]Each rAcTEV protease-treated rBtR contains the residues, GIDPFT, at their amino termini (not shown).

resuspended in 0.01 M HEPES pH 7.4, 0.15 M NaCl, frozen in liquid nitrogen, and stored at −80 °C.

The protein concentration of the BBMV preparation was determined and binding to Cry1Ac was performed by using dot blots as described above. Dot blots were incubated with Cry1Ac protoxin (0.01 μM) and either rCR8-MPR (0.01-, 0.1-, or 0.5 μM) or rCR11 (0.1- or 1 μM) as competitors.

3. Results

3.1. Production and purification of recombinant proteins

From transformed *E. coli*, we purified nine recombinant BtR fragments (rBtRs) corresponding to different portions of the extracellular region (Fig. 1). All rBtRs were insoluble and found in *E. coli* inclusion bodies. Purified rBtRs were obtained in milligram quantities from 1 l bacterial cultures. The expressed proteins contained additional residues at their amino-termini, including the six-histidine tag and a TEV cleavage site, which were removed by treatment with rAcTEV protease (Fig. 1B and Table 3). The cleaved rBtRs were separated from the rAcTEV protease and the amino-terminal tags by Ni^{2+}-affinity chromatography and collecting the unbound protein fractions. Removal of the 27-residue tag by rAcTEV protease resulted in loss of 3.4 kDa from each original peptide. The calculated masses of rBtRs before rAcTEV protease cleavage agree with their actual sizes, as determined by SDS–PAGE and Coomassie blue staining (Fig. 1B and Table 3). The masses of rAcTEV protease-treated rBtRs also were consistent with predicted values, with the exception of rCR7, rCR10, and rMPR, which all were slightly less than the predicted molecular mass based on their gel migration pattern. Minor contaminating protein bands present in some of the samples may be an indication of degradation of the major protein (Fig. 1B).

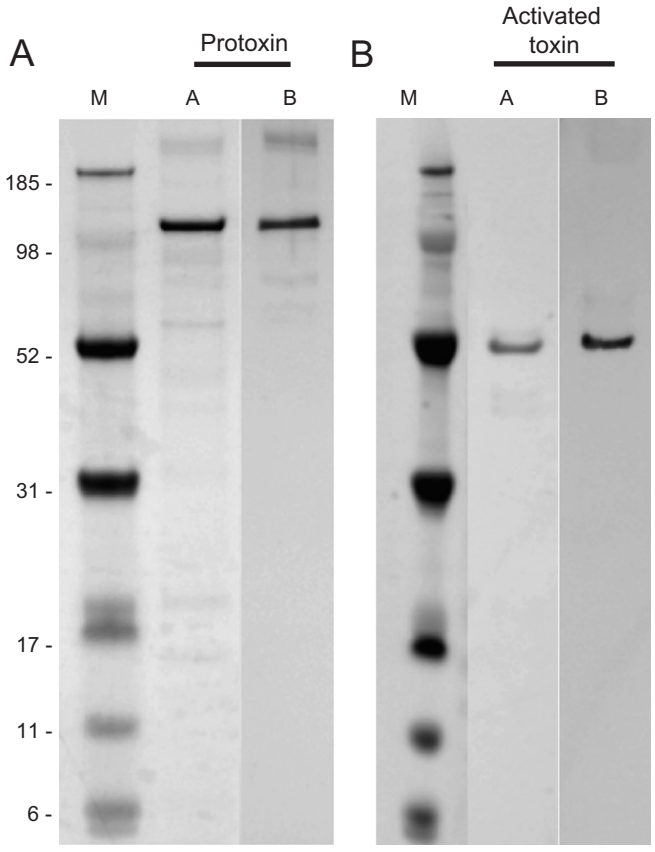

Fig. 2. SDS–PAGE and immunoblot of protoxin and trypsin-activated Cry1Ac. Cry1Ac protoxin and trypsin activated toxin were analyzed on SDS–PAGE gels stained with Coomassie Blue (A) and on immunoblots probed with anti-Cry1Ac sera (B). Lane M shows molecular weight standards (kDa).

The Cry1Ac protoxin used in binding experiments was determined to be free of active toxin (Fig. 2). Active toxin was produced from trypsin-treated protoxin and purified by anion exchange chromatography (Fig. 2).

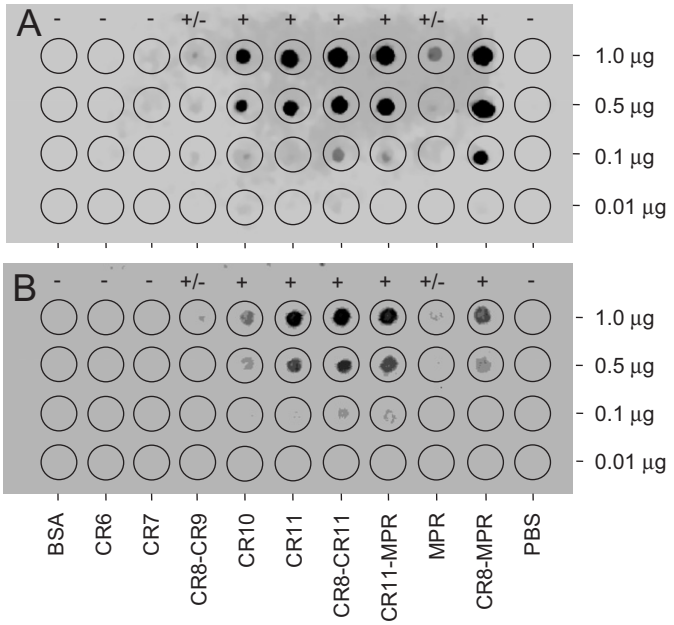

Fig. 3. Dot blot binding of rBtRs to Cry1Ac: toxin overlay. Truncated rBtRs (0.01, 0.1, 0.5, and 1 µg) were dried onto PVDF membrane and probed with Cry1Ac antibodies for binding to 10 nM Cry1Ac (A) protoxin and (B) trypsin-activated toxin. Negative controls (BSA and PBS buffer) are shown.

3.2. Dot blot binding of rBtRs to Cry1Ac: toxin and rBtR overlays

With rBtRs immobilized and toxin overlaid, Cry1Ac protoxin and activated toxin bound to rCR10, rCR11, rCR8–CR11, rCR11-MPR, and rCR8-MPR (Fig. 3). For these peptides, as the amount of immobilized rBtR increased, the intensity of staining indicative of Cry1Ac detection increased. Weak binding was detected to both forms of Cry1Ac for rCR8–CR9 and rMPR, whereas no binding was detected for rCR6, rCR7, or negative controls.

With Cry1Ac immobilized and rBtRs overlaid, binding to Cry1Ac protoxin was detected for rCR8–CR9, rCR8–CR11, rCR11-MPR, rCR8-MPR, and rMPR (Fig. 4). For these peptides, the intensity of staining, indicative of rBtR detection, increased as the amount of rBtR increased. No binding was detected for rCR11. However, antiserum made against rCR8–CR11 recognized rCR11 weakly (Table 2) and might have been insufficient to detect bound rCR11 (Fig. 4). Faint spots were observed for rCR8–CR11 and rCR8-MPR to 1 µm trypsin-activated Cry1Ac, whereas no binding was detected for rCR8–CR9, rCR11, rCR11-MPR, or rMPR. Binding of toxin to rCR6, rCR7, and rCR10 was not tested by this method because available antisera did not recognize these peptides (Table 2).

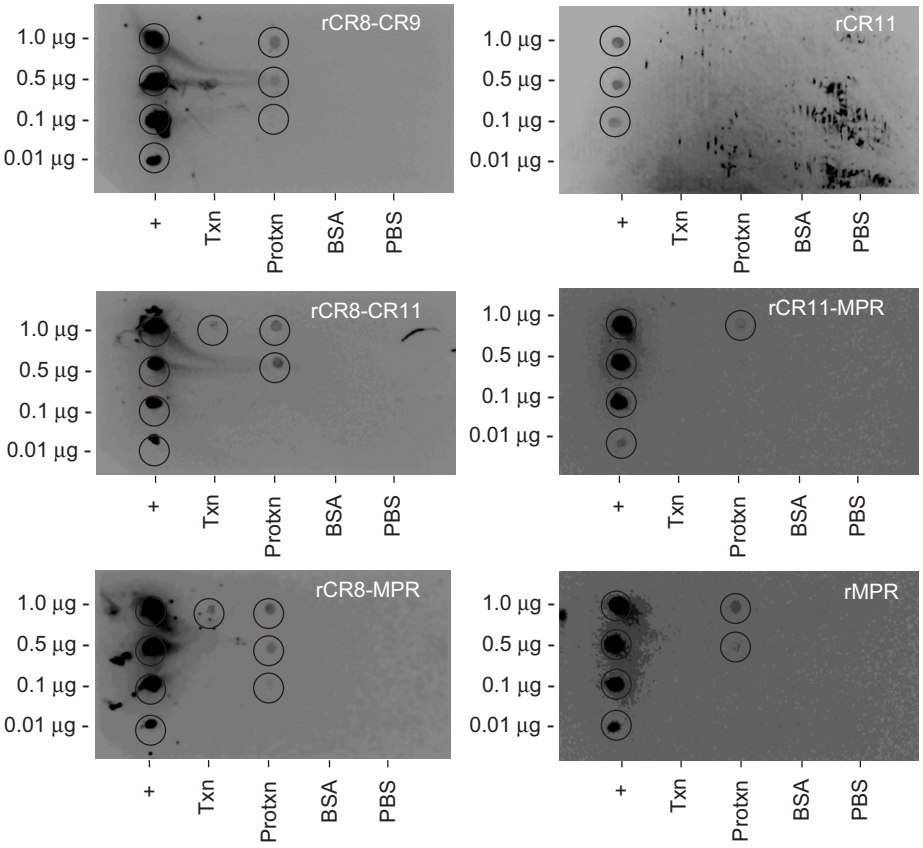

Fig. 4. Dot blot binding of rBtRs to Cry1Ac: rBtR overlay. Cry1Ac protoxin (Protxn) and trypsin-activated toxin (Txn) (0.01, 0.1, 0.5, and 1 µg) were dried onto PVDF membranes, incubated with 10 nM rBtR, and probed with BtR-specific polyclonal antibodies (see Methods for details). For positive controls (+), rBtRs were spotted directly onto membranes and probed with antibodies. Negative controls (BSA and PBS buffer) are also shown. Binding is shown by presence of circle.

3.3. Ligand blot binding of rBtRs to Cry1Ac

In ligand blots (Fig. 5), no binding was detected to Cry1Ac protoxin or toxin by rCR6, rCR7, rCR8–CR9, rCR10, rCR8–CR11, or MPR. rCR11, rCR11-MPR, and rCR8-MPR bound to Cry1Ac protoxin (Fig. 5A), whereas only rCR11 and rCR11-MPR bound to trypsin-activated Cry1Ac (Fig. 5B). Interestingly, rCR8-MPR bound protoxin (Fig. 5A), but not trypsin-activated toxin (Fig. 5B).

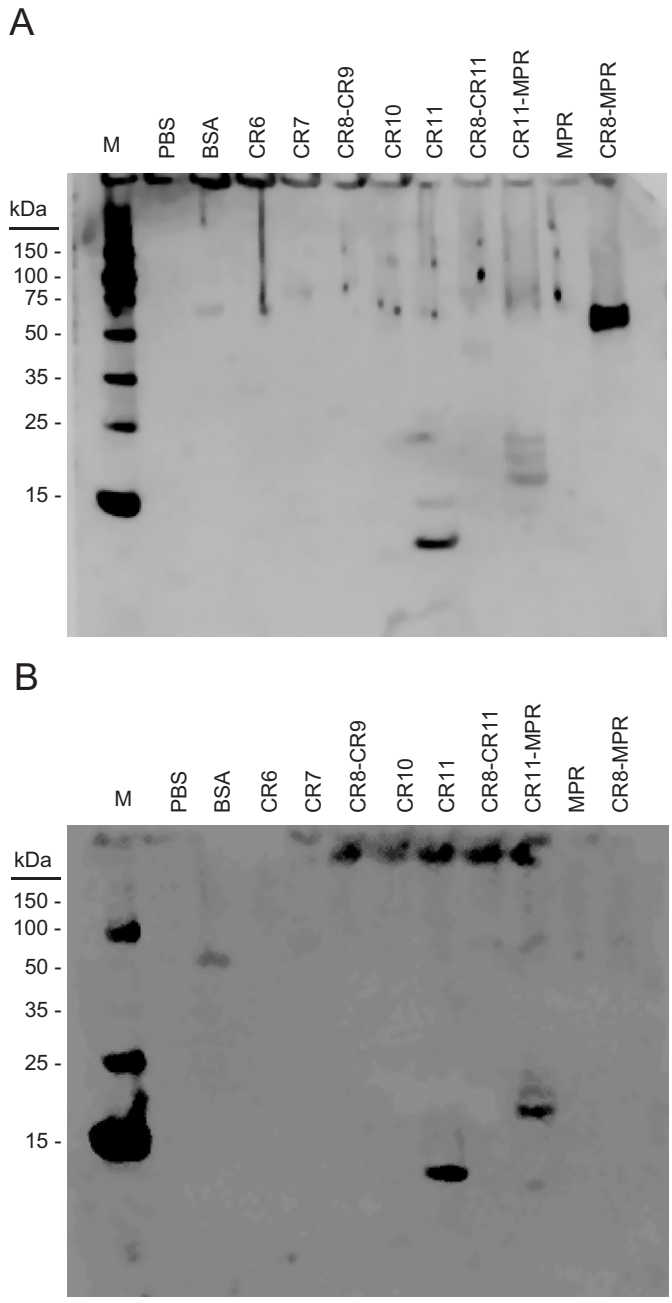

Fig. 5. Ligand blot binding of rBtRs to Cry1Ac: toxin overlay. rBtRs (1 μg) were separated by SDS–PAGE and transferred to PVDF membranes. Ligand blots were overlaid with Cry1Ac (A) protoxin or (B) trypsin-activated toxin. Binding was detected with Cry1Ac antibodies. Negative controls (PBS buffer and 1 μg BSA) are also shown. Lane M shows molecular weight standards.

In the ligand blots, rBtRs were denatured, which could either enhance or diminish binding of Cry1Ac relative to binding with native peptides.

3.4. Dot blot binding of BBMVs to Cry1Ac and competition with rBtRs

In dot blot assays, binding of Cry1Ac protoxin to BBMVs from PBW midguts increased as BBMV concentration increased (Fig. 6A and C). Trypsin-activated Cry1Ac also bound to BBMVs, but binding was weaker for toxin than protoxin (data not shown). When 0.1 or 1 μM of rCR11 was overlaid with Cry1Ac, binding of BBMVs to the protoxin was diminished (Fig. 6B and D), indicating that the CR11 region of PBW cadherin is involved in toxin binding. No competition for toxin binding to BBMVs was observed when 0.01, 0.1, or 0.5 μM rCR8-MPR was overlaid with Cry1Ac (data not shown). Binding of protoxin to immobilized rCR11 was also reduced by addition of rCR11 as competitor, although inhibition was not complete (compare Fig. 6A–D).

4. Discussion

Our results show binding of Bt toxin Cry1Ac to recombinant peptides corresponding to several portions of PBW cadherin protein BtR, including CR8–CR9, CR10, CR11, and MPR. The multiple binding sites for Cry1Ac seen in PBW BtR are similar to findings with cadherin and Cry1A toxins from other Lepidoptera (Fig. 7). Both BtR_1 from *Manduca sexta* and BtR_{175} from *Bombyx mori* contain at least three toxin binding sites. Cadherins from PBW, *H. armigera*, *B. mori*, *M. sexta*, and *H. virescens* have a major Cry1A toxin binding site in the CRs immediately adjacent to the MPR (Fig. 7). Cry1Ac bound particularly well to the larger rBtR fragments (rCR8–CR11, rCR11–MPR, and rCR8-MPR) (Figs. 3–5), suggesting that these peptides contain multiple binding sites and that cooperative binding may be involved.

In dot blot binding assays with either toxin or rBtRs immobilized, Cry1Ac protoxin and toxin bound to the same rBtR fragments except for rCR10 and rCR11 (Figs. 3 and 4). rCR10 and rCR11 bound to Cry1Ac when immobilized to dot blot (Fig. 3), but binding was not detected when Cry1Ac was immobilized due to lack of antibody cross-reactivity to rCR10 or rCR11. Although weak, toxin bound to rCR8–CR9 and rMPR in dot blot assays (Figs. 3 and 4). These regions have not been previously identified as toxin binding regions in other lepidopteran cadherins. Cry1Ac bound to rCR10 and rCR11 when 5–10 fold less protein was immobilized than for rCR8–CR9 or rMPR (Fig. 3), indicating stronger binding to rCR10 and rCR11 and implicating these regions as important toxin binding sites. Interestingly, the BtR fragments seem to bind to Cry1Ac protoxin better than to active toxin. However, because the antibody used in binding assays was made against protoxin, the assay was

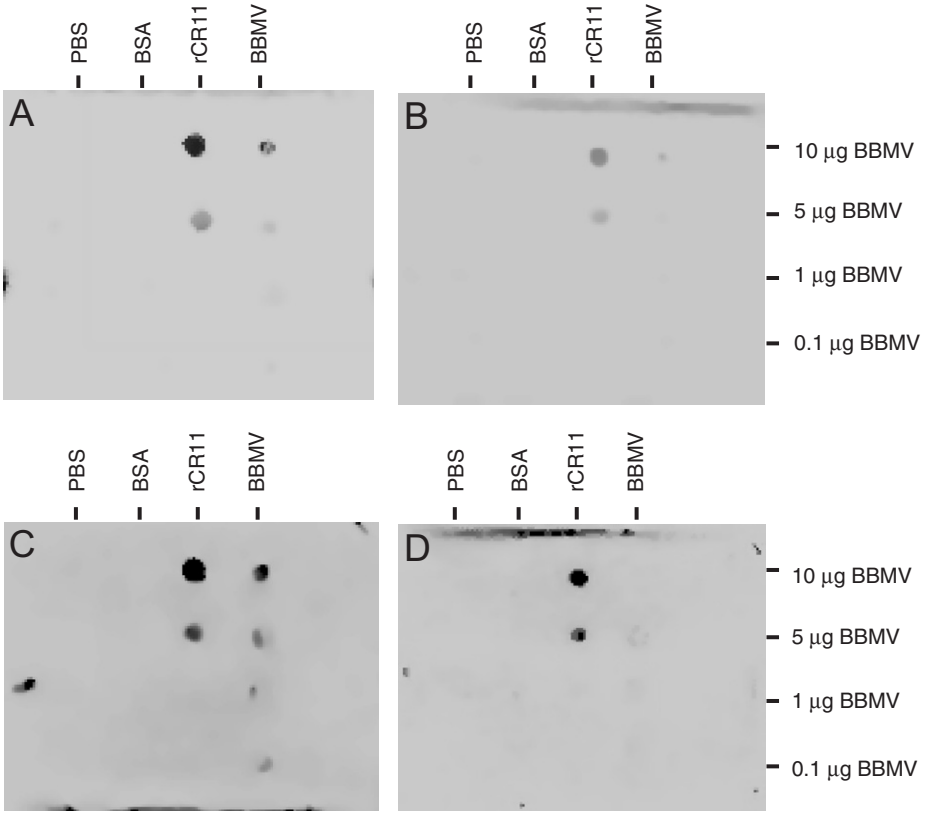

Fig. 6. Dot blot binding of BBMV to Cry1Ac. BBMV proteins (0.1, 1, 5, and 10 µg) were dried onto PVDF membranes and probed for binding to Cry1Ac protoxin (A–D). Blots were incubated with 10 nM Cry1Ac protoxin (A–D) and rCR11 as a competitor at either (B) 0.1 µM or (D) 1 µM rCR11. Blots A and C were incubated with toxin only. Binding was detected with Cry1Ac antibodies. As a positive control, rCR11 (0.01, 0.1, 0.5, and 1 µg) was spotted directly onto membranes. Negative controls (BSA and PBS buffer) are also shown.

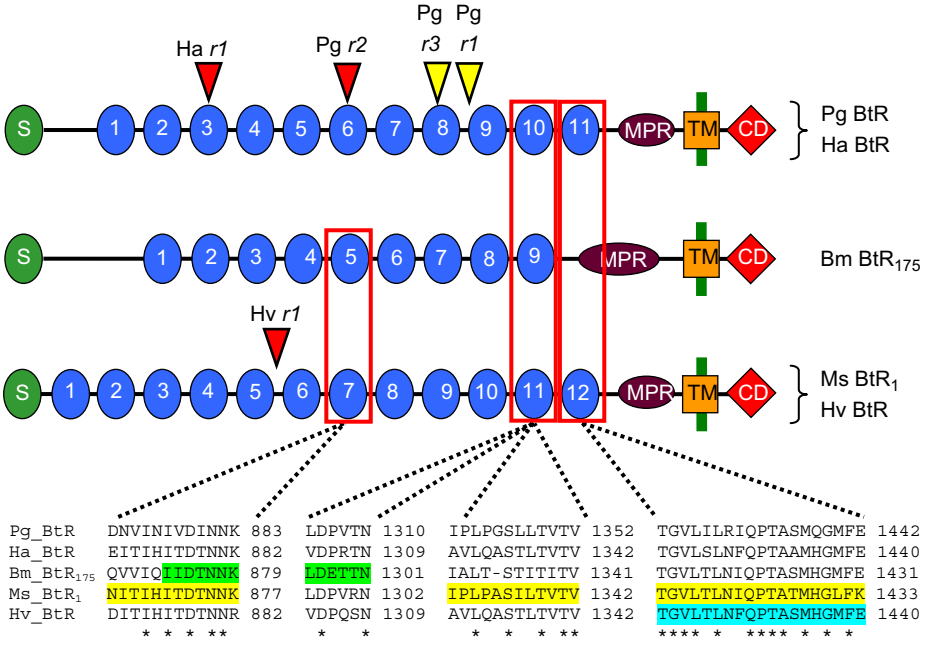

Fig. 7. Cry1A toxin binding sites and resistance-linked mutations in lepidopteran cadherins. Reported binding regions for Cry1A toxins are shown with boxes, with putative binding sequences highlighted (Gomez et al., 2001, 2003; Dorsch et al., 2002; Hua et al., 2004; Xie et al., 2005). Mutations linked with resistance to Cry1A toxins include three in *P. gossypiella* (Pg *r*1, *r*2, and *r*3), one in *H. armigera* (Ha *r*1), and one in *H. virescens* (Hv *r*1). All five mutant cadherins are predicted to be shortened proteins, including three with stop codons (red triangles) that lack toxin-binding regions (Pg *r*2, Ha *r*1, Hv *r*1). In Pg *r*1 and *r*3 (yellow triangles), deletions upstream of toxin-binding regions are predicted to omit 8 and 42 amino acids, respectively.

probably more sensitive to protoxin than to active toxin. Quantitative binding experiments are needed to more rigorously assess the binding affinity of rBtRs to protoxin versus active toxin.

Our results indicate that rCR11 can bind to Cry1Ac under both native and denaturing assay conditions, unlike CR12 from Bt-R_{1a} in *M. sexta* which bound Cry1Ab in dot blots and saturation assays, but not on ligand blots (Hua et al., 2004). Gomez et al. (2001, 2003) identified a Cry1Ab binding epitope ^{870}HITDTNNK^{877} in CR7 of *M. sexta* Bt-R_1 and a similar epitope, ^{873}IIDTNNK^{879}, in CR5 of *B. mori* BtR_{175} that bound Cry1Aa (Gomez et al., 2002a, b). We did not observe binding of Cry1Ac to rCR6, even though PBW BtR has the amino acid sequence, ^{877}IVDINNK^{883}. Rather, our results with PBW BtR CR6 are similar to results from *M. sexta* Bt-R_1, in which no toxin binding was detected in this region (Dorsch et al., 2002; Hua et al., 2004). Gomez et al. (2002a, b) identified novel toxin binding regions in *B. mori* BtR_{175} CR9 (^{1296}LDETTN^{1301}) and *M. sexta* Bt-R_1 CR11 (^{1331}IPLPA-SILTVTV^{1342}). In our dot blot experiments, rCR10 bound to Cry1Ac. rCR10 contains the sequences ^{1305}LDPVTN^{1310} and ^{1341}IPLPGSLLTVTV^{1352}, which are probably Cry1A toxin binding domains in PBW BtR.

Incubation of BBMV proteins with rCR11 resulted in reduced binding to Cry1Ac protoxin, indicating that this region is important for toxin binding in the insect gut. Interestingly, rCR11 did not completely inhibit binding. This result may be explained by several possible scenarios, including (1) additional binding sites exist in native cadherin that are distinct from CR11, (2) novel receptors other than cadherin exist in BBMVs, (3) the total number of CR11 binding sites in BBMV may be larger than the amount of competitor added, and/or (4) at least some binding of toxin to the BBMVs may be irreversible. Furthermore, because rCR11 added as competitor did not completely inhibit binding of protoxin to immobilized rCR11, the amount of competitor probably was not sufficient to saturate all possible binding sites. rCR8-MPR contains CR10 and CR11 toxin binding regions, but did not inhibit toxin binding to BBMVs at the tested concentrations. It is possible that the concentration of competitor peptide was also insufficient to visualize a reduction in binding.

The binding results with recombinant peptides do not necessarily correlate with toxicity in live insects, but independent evidence shows that disruption of cadherin can block binding and toxicity. At least five different mutant alleles of cadherin proteins are linked with resistance to Bt toxin Cry1Ac: one in *H. virescens*, one in *H. armigera*, and three in PBW (Gahan et al., 2001; Morin et al., 2003; Xu et al., 2005) (Fig. 7). The *r*1 mutation in *H. virescens* results in truncated BtR-4 and interferes with binding of Cry1Aa and toxicity of Cry1Ac (Gahan et al., 2001; Jurat-Fuentes et al., 2004). Cadherin from *H. virescens* altered by site-directed mutagenesis had reduced binding affinity to Cry1Ac and reduced ability to inhibit toxicity of Cry1Ac (Xie et al., 2005).

Each of the three mutations in PBW (*r*1, *r*2, and *r*3) is expected to cause loss of at least eight amino acids from the extracellular region of BtR (Morin et al., 2003). The *r*2 mutation in PBW, *r*1 mutation in *H. armigera*, and the *r*1 mutation in *H. virescens* all introduce premature stop codons predicted to result in synthesis of incomplete BtRs (Fig. 7). The *r*1 and *r*3 deletions from PBW occur in regions corresponding to CR8 of BtR and result in proteins with intact binding regions and loss of only 8 and 42 residues, respectively (Fig. 7). This suggests that the regions of BtR affected by *r*1 and *r*3 may be important for structural integrity or other functions, but appear not to be directly involved with binding of Cry1Ac. Further studies on the mutant BtRs should reveal how these mutations affect toxin binding and toxicity.

We report that an insect cadherin binds to both the protoxin and trypsin-activated toxin forms of Cry1Ac. It is unknown if binding to one or both forms of the toxin leads to greater toxicity. This result does suggest that Bt toxin activation could occur either before or after receptor binding and that the activation status of the Bt toxin could influence the mechanism and efficiency of cytotoxicity. One hypothesis for Cry toxin mode of action involves insertion of oligomers into the midgut apical membrane that form pores and cause cytotoxicity (Schnepf et al., 1998; de Maagd et al., 2001). Our results show binding of BtR fragments to Cry1Ac protoxin and support the model involving the formation of a pre-pore oligomer as a prerequisite for membrane insertion (Gomez et al., 2002a, b; A. Bravo and M. Soberon, personal communication). Nonetheless, we do not know if Cry1Ac protoxin interacts with midgut cells. If cadherin initiates a cell-death signal transduction pathway as proposed by Zhang et al. (2005,2006), then binding of monomer is critical and cytotoxicity might differ between toxin and protoxin. Further studies on binding interactions between BtR and the different forms of Cry1Ac (activated versus protoxin and monomer versus oligomer) should provide insight into how cadherin mediates toxicity.

Acknowledgments

We thank Luke Masson and Anthony Pang for providing Cry1Ac toxin and Cry1Ac antisera, respectively. We acknowledge Aaron Gassmann, Shai Morin, and Brenda Oppert for helpful comments on an earlier version of this manuscript. We are grateful to Tim Dennehy and Yves Carrière for helpful discussions and Barbara Hefner for laboratory assistance and insect rearing. This is a cooperative investigation between USDA-ARS and the University of Arizona.

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